PEST MANAGEMENT GRANTS FINAL REPORT

Title

BEHAVIOR MODIFICATION IN NEMATODE MANAGEMENT

Agreement Number

01-0204C

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Statement

Prepared for the California Department of Pesticide Regulation

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Acknowledgements

We acknowledge with gratitude the technical assistance of Patrick Riggs, Tama Fox, Renuka Narayan and Tabitha Santoso.

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Executive Summary

This project was originally funded for a two-year period but was reduced to a single year due to the budget crisis of the State of California. With the seed money provided by one year of funding, we have developed a reliable assay for "attractant and repellent" compounds from root exudates. The assay is sufficiently robust to accommodate behavioral responses that are taxes, kineses or both.

With the current funding, our approach has been mainly reductionist. We have tested individual extract components, primarily flavonoid and phenolic compounds. Since the attractant signal of roots to nematodes could be a mixture of molecules, we are currently testing the effect of combinations of molecules of different chemical structure, which may be detected by the nematode at different chemoreceptor sites. Both attractant and repellent compounds are of interest. Attractants allow the strategy of confusing nematodes by providing an array of blank signals; repellents suggest the possibility of negating attractant forces in root exudates. Any signal that either confuses nematodes so that they are unable to find plant roots, repels them from roots, or attracts them to unfavorable regions of the soil will potentially provide strategies that are alternatives to the use of large volumes of toxic pesticides in soil systems.

With the unavailability of Ca DPR funds, this research will be continued using funds from other sources, but at a lower level of activity.

Introduction

Nematodes cause an estimated US$100 billion in damage to agricultural crops worldwide (Oka et al, 2000). Control with nematicides has serious drawbacks, including short-term management problems and long-term environmental problems. Since the target organisms are dispersed through the soil volume and their precise location unknown, large volumes of chemical are applied with most of the toxicant molecules never encountering target nematodes. A solution is to have the nematodes move to the nematicide using the same principles of chemotaxis by which they so effectively locate plant roots. Discrete, terminal bait stations, *i.e.* traps, containing limited amounts of natural products, or even conventional nematicides, would be preferable to most current nematode management schemes. To achieve this goal, however, fundamental information on the chemical control of nematode behavior is needed. By defining the natural chemical forces operating on nematodes, we can help develop both short-term solutions in the form of a trap and longer-term solutions based on plant genetics.

Objectives

1. Identify nematode behavioral cues (*e.g.* attractants or repellents) in root exudates.
2. Identify new natural nematicidal compounds.
3. Combine a natural attractant and a nematicide into a nematode trap.

Premise

Several categories of natural products are associated primarily with plants and are identified in root exudates (Table 1) (Graham, 1998). They include amino compounds, organic acids, carbohydrates, phenolics, flavonoids, enzymes, nucleotides, chalcones, fatty acids, sterols and other miscellaneous compounds. Of these, flavonoids and phenolics seemed likely candidates as plant signature compounds for nematodes due to their known effects on other organisms, both as attractants and defense mechanisms. They were selected for initial study.

Table 1: Root exudate components of various plants with references to information sources (adapted from Graham, 1998).

Amino-Compounds	Organic Acids
&-alanine; (8,16,23,28,45,46,47,48,50)	oxalic acid; (8,37,46,47)
asparagine; (8,16,23,28,46,50)	malic acid; (8,21,22,24,25,28,28,30,37,46,47)
leucine/isoleucine; (8,16,23,28,46,47,48,50)	acetic acid; (8,46)
valine; (8,16,23,28,46,47,50)	propionoic acid; (8,46)
glutamine; (8,16,23,28,46,50)	butyric acid; (8,46)
serine/homoserine; (8,16,23,28,46,47,50)	valeric acid; (8,46)
glycine; (8,16,23,22,28,46,47,50)	citric acid; (8,11,21,22,24,25,26,28,28,30,37,46,47)
phenylalanine; (8,16,18,23,46,47,48)	succinic acid; (8,24,28,28,30,37,46,47)
threonine; (8,16,23,46,47)	fumaric acid; (8,28,29,37,47)
tyrosine; (8,16,18,26,46,47,50)	glycolic acid; (8,46)
lysine; (8,16,23,22,28,46,47,50)	deoxymugineic acid; (1)
proline; (8,16,23,46,50)	malonic acid; (8)
methionine; (8,16,23,46,50)	2-ketogluconic acid; (38)

cystathionine; (8,46)	tartaric acid; (8,29,37,47)
ornithine; (8,16,23,46,50)	isocitric acid; (37)
citrulline; (23,16)	acolnitic acid; (29,47)
arginine; (8,11,16,28,46)	3-phenyl propionoic acid; (56)
glutamate; (47,48,50)	p-hydroxybenzoic acid; (4,9,41,54,56)
aspartate; (47,48,23,50)	2,5-dihydroxybenzoic acid; (56)
tryptophan; (8,18)	myristic acid; (56)
histidine; (8,23,16,46,47)	p-hydroxycinnamic acid; (52,56)
cysteic acid; (8,46)	palmitic acid; (8,56)
aspartic acid; (8,16,23,28,46)	aconitic acid; (29)
glutamic acid; (8,16,23,22,28,46)	stearic acid; (8,56)
-amino butyric acid; (8,16,28,46)	oxalocetic acid; (29)
amino adipic acid; (16)	uronic acid; (38)
ethanolamine; (16)	glutaric acid; (29)
2,4-dihydroxy-1,4-benzoxazin-3-one; (34)	glyoxylic acid; (29)
ammonium; (37)	pentadecanoic acid; (52)
ammonia; (8)	
cystine; (16,46)	
benzoxazolin-2-one; (34)	
6-methoxybenzolin-2-one; (34)	
2,4-dihydroxy-7-methoxy-1,4-benzoazin-3-one; (34)	
Phenolics	**Carbohydrates**
salicylic acid; (54)	glucose; (8,16,29,38,46,47,48,50)
p-hydroxybenzoic acid; (4,9,41,54)	fructose; (8,16,29,38,46,47,48,50)
vanillic acid; (4,41,54)	maltose; (8,46)
syringic acid; (4,15,52,54)	galactose; (8,46,47)
4-methoxyindole-3-acetonitrile; (54)	ribose; (8,46,47,48)
pyrocatechol; (54)	xylose; (8,38,46,47)
coumesterol; (9, 43,44)	rhamnose; (8,46)
caffeic acid; (18,26)	arabinose; (8,29,46,47)
p-thiocyantophenol; (56)	raffinose; (8,46)
2-hydroxybenzothiazole; (56)	oligosaccharides; (8,46)
3,4-dimethylbenzoic acid; (52)	myo-inositol; (50)
benzoic acid; (18,29,52,56)	deoxyribose; (8)
phenylacetic acid; (52)	sucrose; (8,16,29,47,48,50)
2-methoxyphenol; (52)	deoxysugars; (8)
hydrocinnamic acid; (52)	
cinnamic acid; (18,52,56)	
2-methoxy phenylacetic acid; (52)	
3-hydroxy hydrocinnamic acid; (52)	
4-hydroxy-3-methoxy hydrocinnamic acid; (52)	
4-hydroxy-2-methoxycinnamic acid; (52)	
ferulic acid; (4,6,14,18,52)	
cyclopropyl-p-benzoquinone (14)	
2,6-dimethoxy-p-benzonquinone (14)	
tetrafluorbenzoquinone (14)	
benzoquinone (14)	
SXSg (14)	
strigol (14)	
resorcinol (14)	
dihydroquinone (14)	
sinapic acid; (15,52)	

2-(3',5'-dihydroxyphenyl)-5,6-dihydroxy-benzofuran; (34)	
Flavonoids	**Enzymes, Nucleotides & Chalcones**
kievitone; (26)	invertase; (46,8)
4',7-dihydroxyflavone; (9,12,15,19,35,36,44)	amylase; (46,8)
4',7-dihydroxyflavanone; (9,12,15,19,35,36,44)	protease; (46,8)
formononetin-4',7-dihydroxyflavonone; (9,19,35,36,44)	guanine; (46,8)
4',5,7-dihydroxyflavonone; [apigenin] (9,18,26,43)	adenine; (46,8)
apigen-7-0-glucoside; (9,15)	polygalacturonase; (8)
genistein; (15,17,18,43)	phosphatase; (7,8)
3',4',5,7-tetrahydroxyflavone; [leuteolin] (9,15,18,15,26,42,43)	uridine/cytidine; (8)
4',7-dihydroxyisoflavone; [daidzein] (9,15,17,18,43,44)	4,4'-dihydroxy-2'-methoxychalcone; (10,19,35,36)
3,4',5,7-tetrahydroxy flavone; [kaempferol] (9,15,18,26,43)	**Fatty Acids & Sterols**
coumestrol; (9,43,44)	cholesterol; (8)
formononetin-7-0-(6"-0-malonylglucoside) ; (9,10)	palmitic acid; (8)
formononetin; (9,14,18,36,44)	-sitosterol; (8,50)
3',4',7-trihydroxyflavone; (9,15)	stigmasterol; (8,50)
4'7-dihydroxy-3'methoxflavone, [geraldone] (9,12,44)	campesterol; (50,8)
4'-hydroxy-7-methoxyflavone; (9,44)	stearic acid; (8)
xenognosin A & B (14)	oleic acid; (8)
	linoleic acid; (8)
	fatty acids 18:1; 18:2; 18:3; 20:0; 22:0; 24:0; (50)
Miscellaneous Compounds	
epi-3-hydroxy-mugineic acid; (2,45,53)	medicarpin-3-0-glycoside; (8,10)
8-methylsulfinyloctyl isothiocyanate [histurin]; (54)	umbelliferone; (9,43,44)
benzyl isothiocyanate; (51,54)	coumarins; (4,9,41,43)
auxins; (8,32)	nodulation gene inducers; (8,43)
scopoletin; (8,41)	assorted allelopathic compounds; (6,8,55)
fluorescent substances; (8)	metal chelators; (8)
vitamins; (8)	ethanol; (47)
hydrocyanic acid; (8)	methanol; (8)
glycosides; (8)	formaldehyde; (8)
saponin; (8)	acetaldehyde; (8,48)
organic phosphorous compounds; (8)	proionaldehyde; (8)
nematode cyst or egg hatching factors; (8,46)	acetone; (8)
nematode attractants/nematicides; (8,46)	ethylene; (8)
fungal mycelium stimulants and inhibitors; (5,8,13)	propylene; (8)
zoospore attractants; (5,8,33,46)	various volatiles; (3,5)
spore and aclerotium germination stimulants and inhibitors; (5,8,39)	gibberellins; (8, 18)
parasitic weed germination stimulants; (8,39)	cytokinins; (8)
medicarpins; (8,10,34)	

Our preliminary tests suggested that *Meloidogyne javanica* juveniles are significantly attracted to 4',7-dihydroxyflavone at 100 nM concentration, however, that result proved difficult to duplicate in subsequent tests (Table 2), which led us through an evolution of methodology.

Results

Preliminary Methodology Tests

Racetracks

In previous and initial studies, we conducted chemotaxis assays on "nematode racetracks", grooved microscope slides which accommodate strips of 1% agar, modeled after a design by Castro et al. (1989). Nematodes are concentrated in a groove where, in principle, they can only move in two directions: toward or away from the test compound. In practice, we noted a strong tendency for nematodes to move upward, out of the groove, and aggregate on the dry slide surface, even if covered with a coverslip. After several months of attempting to modify the protocol, we abandoned this approach as unreliable.

Center spots

A second methodological iteration was to place the nematodes in the center of a petri dish, the test solution at one side and a control solution at the other. Arcs were inscribed on the base of the dish with radii centered on the test and control spots. Nematode abundance in each arc was determined periodically. A problem with this method was apparent lack of detection of the signal, or lack of response to the signal, across the radius of the petri dish. The majority of nematodes remained close to the center of the dish.

Line transects

In this method, the test chemical was mixed into melted agar and poured into the dish. After setting, the agar on one side of the dish was removed and replaced with agar not treated with the chemical. A groove was melted across the surface of the agar at the interface. Nematodes were distributed across the groove and their abundance in both halves of the dish was determined periodically. This method attempted to build on our observation that nematodes tend to climb out of a groove (see racetrack method) and to recognize that the response to the stimulus could be either a kinesis or taxis or both. So, nematodes climbing out of the groove may undergo different kinetic behavior on either side of the dish and remain in that sector or move out.

Table 2. A summary of tests of flavonoids as chemical attractants of nematodes using the *racetrack, center spot* and *line transect* methods. Attraction percent is the abundance of nematodes in proximity of the test chemical relative to those in proximity of a water control, discounting nematodes that did not move. The data were quite variable, differences usually not significant, and not consistent among different trials.

Meloidogyne Data		**C. elegans Data**	
Compound	Attraction percent	Compound	Attraction percent
Eriodoctyol	42.0	Quercitin	86.0
Genistein	31.7	4,7dihydroxyflavone	77.7
Formononetin	50.0	Luteolin	68.9
Coumestrol	33.5	Coumestrol	80.5
Genistein 50µm	39.4	Naringenin	73.2
Genistein 10µm	47.2	4,7 dihydroxyflavanone	80.4

Genistein 1µm	41.8	Trihydroxychalcone	69.9
Genistein 0.1µm	66.9	Eriodictyol	75.8
Genistein 0.01µm	64.7	Formononetin	60.9
Naringenin	47.8	Genestein	72.6
Trihydroxychalcone	56.4	Genestein	56.9
Formonetin	54.5		
Luteolin	38.6		
4,7dihydroxyflavone	45.2		
Quercitin	37.7		
4,7 dihydroxyflavanone	57.9		

Arena Assembly Method Testing

A taxis is directed movement of an organism towards or away from a stimulus. A kinesis is a change in the rate of activity, or frequency of turning of an organism in the presence of a stimulus. Reduced activity or more frequent turning can result in aggregation near the stimulus. All of the above methods test primarily for taxes as the nematodes start at some point remote from the chemical. We recognized that kinetic responses might also be involved, which resulted in subsequent modification of technique.

The arena assembly method is our current protocol (Fig. 1). It tests both tactic and kinetic responses. Concentric circles are scored on the base of a petri dish at 1.5 and 3.5

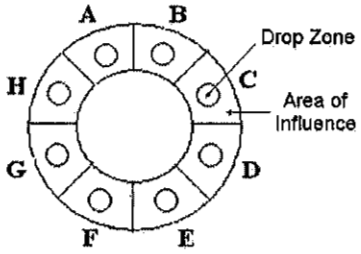


Fig. 1. Arena assembly template for testing tactic and kinetic responses of nematodes to root exudates and substances of plant origin.

cm from the center. Eight circular "drop zones" are scribed, equal intervals apart, within the two circles. The drop zone circles are separated by eight lines radiating from the center of the dish to form sectors of "areas of influence" around each drop zone. Nematodes are distributed on the surface of the dish and allowed sufficient time to move randomly. Initial concentrations of nematodes in each drop zone and arena of influence are determined. The desired concentrations of test chemicals and controls are spotted in 5µl in the drop zones.

Dishes are sealed with parafilm to prevent further drying and placed in the dark. Nematode abundance in the drop zones and arenas of influence are determined at 1, 2 and 4 hours after application of the chemical.

A conceptual response series provides a basis for interpretation of tactic and kinetic behavioral responses observed through the arena assembly method (Fig. 2).

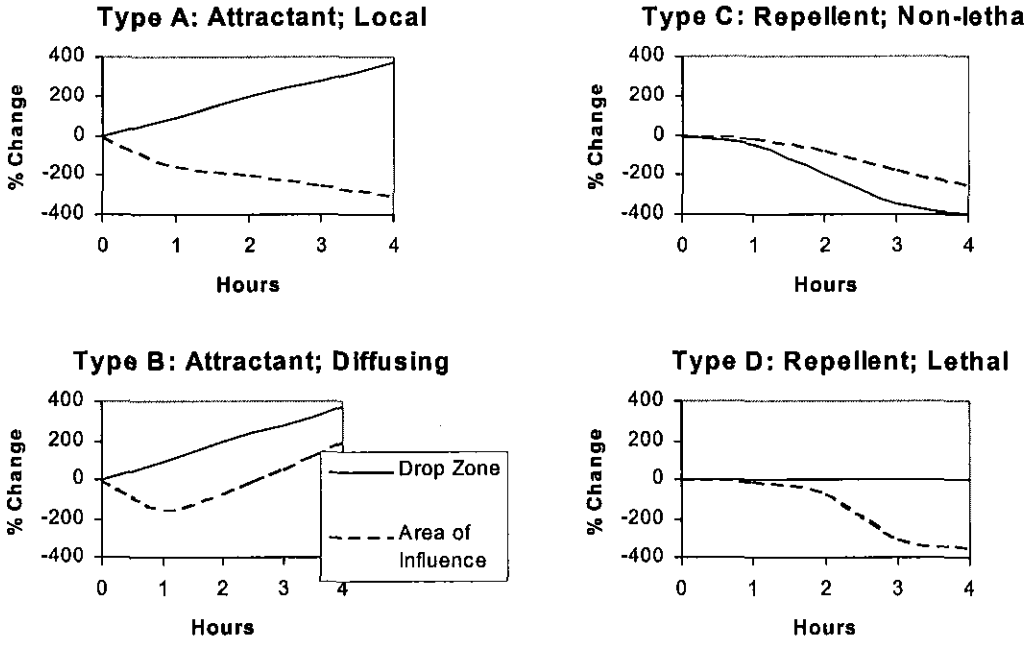
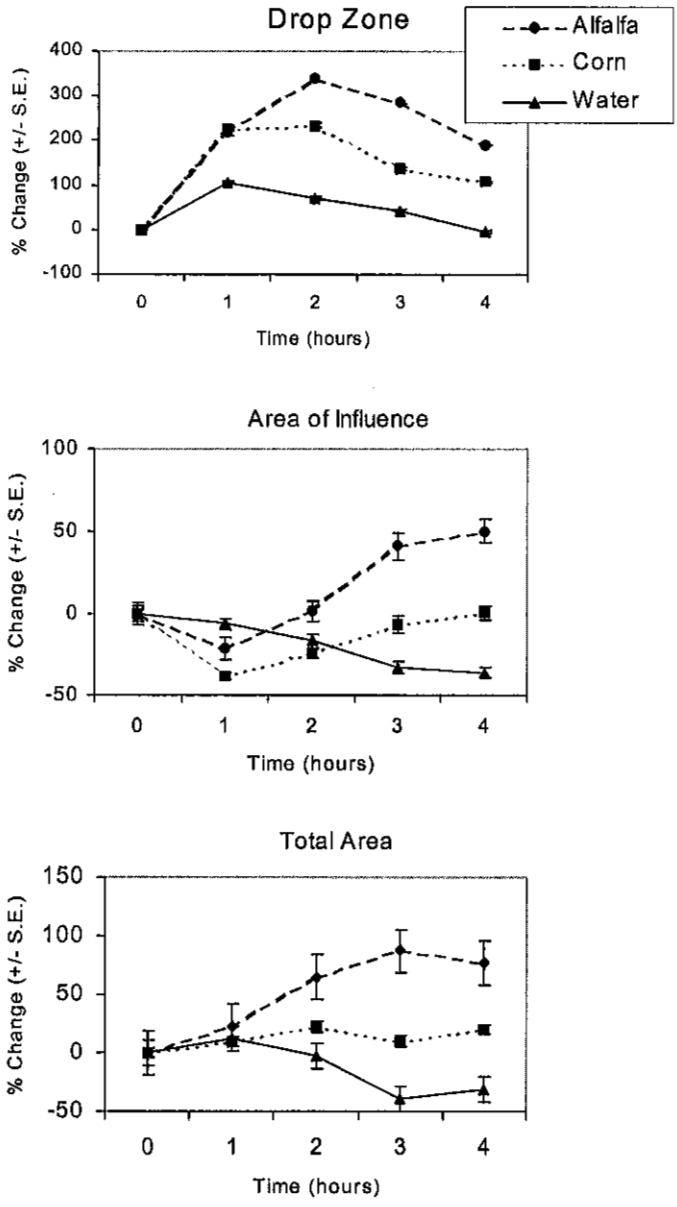


Fig. 2. Possible response patterns of nematodes to chemicals placed in the drop zone (Fig. 1) of the arena assembly template. Type A: There is a localized attractant effect of the chemical in the drop zone, but the effect does not spread over time; nematodes from the area of influence are attracted into the drop zone. Type B: The attractant material in the drop zone gradually diffuses; nematodes in the area of influence initially decrease in number as they emigrate to the drop zone but are later replenished from surrounding areas as the attractant spreads. Type C: Nematodes move away from both the drop zone and the area of influence. Type D: Nematodes in the drop zone are killed by contact with the repellent, those in the area of influence move away. Not shown is the no-response pattern where there is no response to the material and nematode abundance in the drop zone and area of influence does not change.

Sample Results

Juveniles of *Meloidogyne javanica* are attracted to sterile root exudate of alfalfa (*Medicago sativa*) and corn (*Zea mays*) using the arena assembly method (Fig. 3). They also appear to be attracted to the water-soluble vitamin biotin, which acts as a cofactor for enzymes involved in carboxylation reactions, and to a lesser extent to the flavonoid, 4,7-dihydroxyflavanone (Fig. 4). Under the hypothesis that more than one component of root exudates may be necessary to attract nematodes to plant roots, we tested the effect of a combination of biotin and 4,7-dihydroxyflavanone; the attractant effects of the chemicals individually were negated (Fig. 4).

Fig. 3. Temporal and spatial response of juveniles of *Meloidogyne javanica* to sterile root exudate of alfalfa and corn.



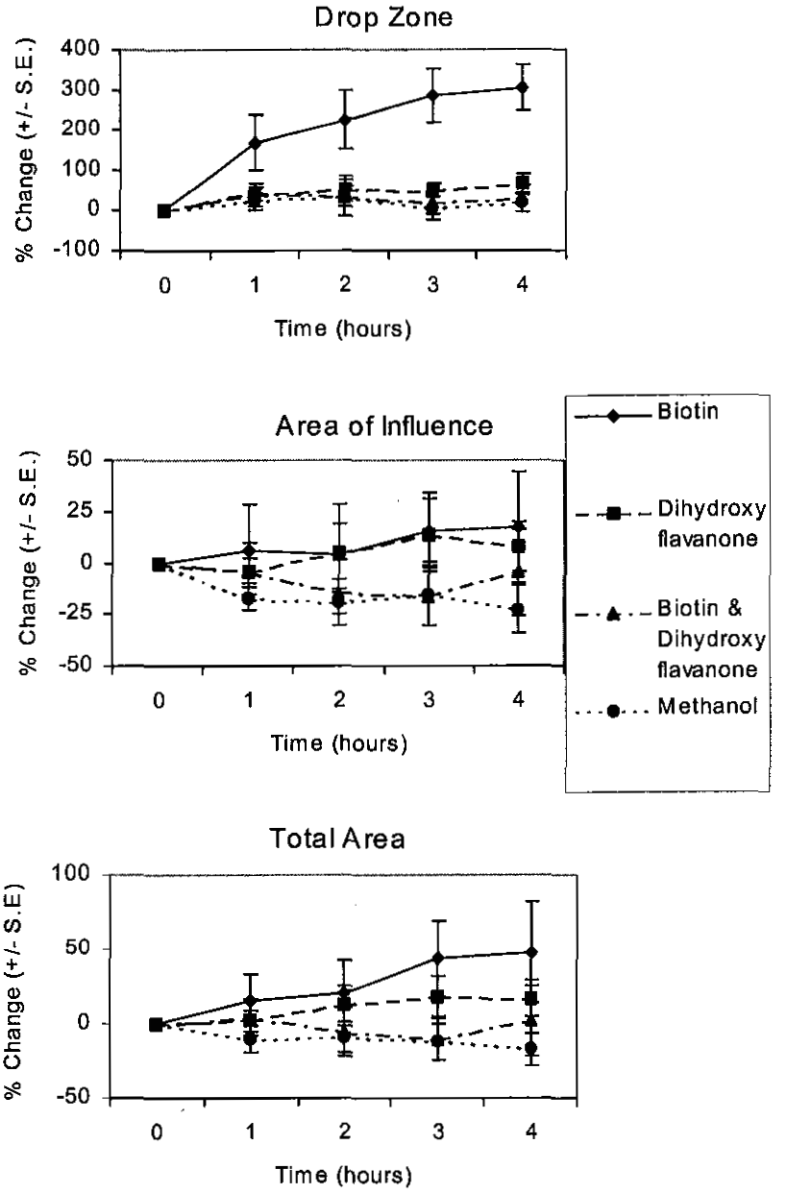


Fig. 4. Temporal and spatial response of juveniles of *Meloidogyne javanica* to biotin, 4,7 dihydroxyflavanone and to biotin and 4,7 dihydroxyflavanone in combination.

Discussion

We believe that we now have a reliable and repeatable assay for "attractant and repellent" compounds from root exudates that recognizes that both taxes and kineses may be involved.

The development of these methods, our climb up the learning curve, and the disappointment of endless negative results by earlier methodology have "burned out" three technical staff, resulting down-time associated with recruitment and training of new

personnel. Key players are now in place and the methodology seems reliable. We will proceed with the screening of other likely candidates from the root exudate component list (Table 1).

Thus far, our approach has been mainly reductionist. We have been testing individual extract components. Conceivably, the attractant signal of roots to nematodes could be a mixture of molecules. So, in parallel with the screening of individual compounds, we will be taking an analytical approach. We have collected alfalfa root exudate under sterile conditions and will test its affect at various concentrations. Within the next month, we will start the fractionation of root exudate from *Medicago truncatula*. That will allow testing of purified and uncontaminated fractions. Both attractant and repellent compounds are of interest. Attractants allow the strategy of confusing nematodes by providing an array of blank signals; repellents suggest the possibility of negating attractant forces in root exudates.

Summary and Conclusions

The Project Summary Form is not applicable to the activities and progress of this preliminary research. Further, it could not be downloaded into this report. The pertinent requested information follows:

A. Proposal Title: Behavior Modification in Nematode Management.
B. Principal Investigator: Howard Ferris and Donald Phillips.
C. Alternative Practices: Target pests are plant-feeding nematodes; alternative practices are to provide chemical stimuli that confuse nematodes or attract them to repositories of toxins or natural enemies.
D. Summary of Project Successes: The project investigated many approaches to assaying taxes and kineses in nematodes. A reliable arena method was tested and developed. A range of flavonoid and phenolic compounds was tested as attractants and repellants.
E. Number of Participating Growers: Not applicable.
F. Total Number of Acres in Project: Not applicable.
G. Number of Project Acres under Reduced Risk: Not applicable.
H. Total Acres of Project Crop: Not applicable.
I. Non-project Reduced Risk Acres: Not applicable.
J. Number of Participating PCAs: Not applicable.
K. Cost Assessment: Not applicable.
L. Number of Field Days: Not applicable.
M. Attendance at Field Days: Not applicable.
N. Number of Workshops/meetings : Not applicable.
O. Workshop Attendance: Not applicable.
P. Number of Newsletters: Not applicable.
Q. Number of Articles: Not applicable.
R. Number of Presentations: Mentioned in approximately six presentations to grower groups and professional society colleagues..
S. Other Outreach Activities: None.

Note this research is still in the exploratory and development phase. Outreach activities will accelerate later when we have information to extend.

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